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FOUR DIBENZOFURAN PHYTOALEXINS FROM THE SAPWOOD OF *MESPILUS GERMANICA*

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Key Word Index—*Mespilus germanica*; Rosaceae; sapwood; *Nectria cinnabarina*; dibenzofuran phytoalexins.

Abstract—The sapwood of *Mespilus germanica* produced four dibenzofuran phytoalexins when challenged by *Nectria cinnabarina*, a coral spot fungus. The structures of the three new phytoalexins: 6-hydroxy-, 6-methoxy- and 7-hydroxy-6-methoxy-α-pyrufurans were determined with the aid of spectroscopic methods. α-Cotonefuran, found earlier in the genus *Cotoneaster*, was also identified. Their antifungal activities (ED_{50}s) lie in the range of 12–100 ppm, with an average value of 45 ppm.

INTRODUCTION

Mespilus germanica L. is a deciduous tree or shrub in the Rosaceae which is closely related morphologically to *Crataegus* and *Cotoneaster*. It is thought to have originated in southeast Europe to Asia Minor, and is cultivated for the fruits, which are used for preservatives. It was a monotypic species, until the recent discovery in Arkansas, U.S.A. of *Mespilus canescens* Phipps, which has been placed in the same genus on evidence from morphology and isoenzymes [1].

Our previous studies of disease resistance mechanisms in the Rosaceae have revealed that while all six *Cotoneaster* species examined produce one or more of five dibenzofuran phytoalexins in the sapwood [2], both of two *Crataegus* species surveyed contain dibenzofurans as constitutive antifungal agents [3]. It was, therefore, of interest to examine *Mespilus germanica* to determine what disease resistance mechanisms might be present; unfortunately, living material of the geographically distinctive *M. canescens* was not available for study. We here report on the major phytoalexins formed in sapwood of *M. germanica* challenged by the pathogenic fungus *Nectria cinnabarina*.

RESULTS

Although the quantification of these phytoalexins was not carried out, it seems that the amounts produced by this plant were remarkably low. In fact, the presence of dibenzofuran phytoalexins was revealed by UV absorption spectra-orientated fractionation of the extract of diseased tissue; the fungitoxicity was not detected by direct bioassay on developed TLC plates with *Cladosporium herbarum*. In spite of this, the measured ED_{50}s show comparable fungitoxicity with those of other dibenzofuran phytoalexins of this family (see below).

Four phytoalexins were isolated from the sapwood of *Mespilus germanica* following inoculation with *Nectria cinnabarina*, and they were separated and purified with difficulty by repeated TLC. All four compounds were readily recognized as closely related phenolics, from colour reactions and UV spectral analysis (Table 1). On UV and mass spectral comparison with published data, it became apparent that **1** is identical to the dibenzofuran, α-cotonefuran, previously reported as a phytoalexin in five *Cotoneaster* species [2]. This was confirmed by direct comparison with an authentic sample.

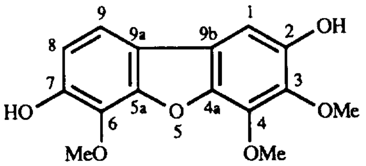
The other three compounds **2–4** appeared to be related benzofurans from their UV, mass spectral and chromatographic properties. They were clearly different from any of the nine known benzofuran phytoalexins, which were available for comparison [2, 3], and hence were new structures. Their identification as 2,6-dihydroxy-1,3,4-trimethoxydibenzofuran (**2**), 2-hydroxy-1,3,4,6-tetramethoxydibenzofuran (**3**) and 2,7-dihydroxy-1,3,4,6-tetramethoxybenzofuran (**4**) followed from an analysis of the NMR data, as discussed below. We have named them 6-hydroxy-α-pyrufuran, 6-methoxy-α-pyrufuran and 7-hydroxy-6-methoxy-α-pyrufuran, respectively, in view of their ready derivation from α-pyrufuran, one of the phytoalexins of the pear tree, *Pyrus communis* [4].

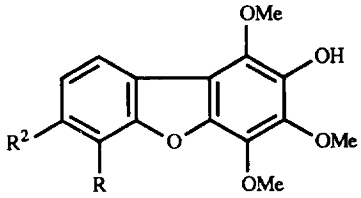
The ^{1}H NMR spectrum of **2** (Table 2) showed signals for three adjacent aromatic protons and three methoxyls. The ^{13}C NMR spectrum (Table 2) revealed 12 aromatic carbons (three methines and nine quaternary) plus three

Table 1. Spectral and chromatographic data for dibenzofurans **2–4**

	λ_{max}	log ε	Alkaline max	EIMS	R_f (× 100)* CA5	HEM	R_t (min)†
6-Hydroxy-α-pyrufuran (**2**)	238	4.17	244	290	22	40	6.4
	268	4.09	340	(100)			
	286	4.03		275			
	310	3.61		(102)			
	321	3.66					
6-Methoxy-α-pyrufuran (**3**)	228sh		234	304	70	51	10.6
	262	3.98	286	(100)			
	285	3.95	322	289			
	306	3.71		(96)			
	318	3.70					
7-Hydroxy-6-methoxy-α-pyrufuran (**4**)	238	4.33	235	320	34	37	6.0
	267	4.20	278	(100)			
	295	4.21	316	305			
	316sh	3.67		(77)			

*On silica gel in CA5 = $CHCl_3$–Me_2CO (19:1); in HEM = hexane–EtOAc–MeOH (60:40:1).
†HPLC on a phenyl column (4 × 250 mm) MeOH–HOAc–H_2O (27:2:20), isocratic, 1 ml min^{-1}.





2, $R^1 = OH, R^2 = H$

3, $R^1 = OMe, R^2 = H$

4, $R^1 = OMe, R^2 = OH$

Table 2. ^1H and ^{13}C NMR chemical shift data for dibenzofurans **2–4**

	^1H			^{13}C		
H/C	**2**	**3**	**4**	**2**	**3**	**4**
1				137.9	138	137.3
2				141.6	142	140.5
3				140.3	—	140.5
4				136.0	136	135.9
4a				142.0	—	142.0
5a				145.6	—	149.2
6				144.1	146.5	133.7
7	6.98 *d*	7.08 *d*		114.3	110.1	149.3
8	7.13 *t*	7.24 *t*	6.91 *d*	124.6	124.9	113.3
9	7.51 *d*	7.63 *d*	7.45 *d*	113.7	115.1	117.2
9a				125.8	126	118.2
9b				115.4	—	115.2
OMe-1	4.01 *s*	4.01 *s*	3.99 *s*	60.8	60.9	60.7
OMe-3	3.94 *s*	3.94 *s*	3.92 *s*	61.8	61.9	61.9
OMe-4	4.13 *s*	4.12 *s*	4.14 *s*	61.4	61.5	61.4
OMe-6		4.03 *s*	4.12 *s*		56.6	61.2

^{13}C resonances for **3** are approximate and were obtained from the ^1H-detected HMBC experiment; insufficient material was available for a ^{13}C-detected spectrum.

methoxyls. Of the quaternary carbons, seven resonating between δ136.0 and 145.6 were oxygenated, and due to their relative shielding it was obvious that there was no isolated oxygenated carbon and that each had at least one adjacent oxygenated carbon. These data were compatible with a 1,2,3,4,6-oxygenated dibenzofuran and left the problem of placing the three methoxyl substituents.

In view of the relative deshielding of the methoxyl carbons (δ60.8–61.8) they must all be sterically hindered [5], so C-6 was ruled out as a methoxylated position. A NOE between the methoxyl at δ4.01 (δ60.8) and the aromatic proton at δ7.51 required placement of one methoxyl at C-1. A second methoxyl, highly deshielded in the H-domain (δ4.13, δ_C61.4) can be attributed to C-4 [2]. Placement of the final methoxyl at C-3 rather than C-2 was based on a comparison with published data for 2-hydroxy-1,3,4-trimethoxydibenzofuran (α-pyrufuran) and 3-hydroxy-1,2,4-trimethoxydibenzofuran (β-pyrufuran) [4]. Both the ^1H and ^{13}C resonances for the methoxyl resonances and the ^{13}C resonances for C-1 to C-5a were much closer to those of α-pyrufuran than β-pyrufuran and indicated that **2** is 6-hydroxy-α-pyrufuran.

A second compound available in very small amounts, gave a ^{1}H NMR spectrum very similar to that of **2** but with an additional methoxyl resonance at δ4.03. The ^{13}C resonance for this methoxyl was at δ56.6 which indicated the presence of an unsubstituted *ortho* position [1]. On this basis it was characterized as 6-methoxy-α-pyrufuran (**3**).

The final compound also had four methoxyl resonances but only two *ortho*-coupled protons, so requiring a further substituent (e.g. 1,2,3,4,6,7-oxygenated dibenzofuran). The deshielded carbon resonances for the methoxyls showed that the C-7 oxygen was not methylated but an HMBC study [6] allowed one to be placed at C-6 (^{3}J couplings of H-8 and the δ4.12 methoxyl protons to C-6 δ133.7), while a NOESY spectrum again revealed an interaction between a C-1 methoxyl and H-9. The remaining signals were comparable with those of **2** and permitted assignment of the structure 7-hydroxy-6-methoxy-α-pyrufuran (**4**).

The three new dibenzofurans **2**–**4** were tested for their antifungal activity against three representative plant fungi, *Alternaria alternata*, *Botrytis cinerea* and *Fusarium culmorum* (Table 3). The ED_{50} values ranged from 12 to 100 ppm, with an average value of 45 ppm. These values are similar to other phytoalexins characterized in the Rosaceae [2, 3]; data for α-cotonefuran and β-pyrufuran are included in Table 3 for comparison. It may be noted that of the three new phytoalexins, **2** is consistently more fungitoxic than **4**, which is likewise consistently more fungitoxic than **3**. Thus, the presence of one free hydroxyl group in both of the aromatic rings of the dibenzofuran structure may contribute towards the antifungal activity of these phytoalexins.

Table 3. Inhibition of spore germination by dibenzofurans in three fungi

a. *Alternaria alternata*

	Germination rate at (μg ml^{-1}) in percent				ED_{50}	
Compound	10	20	50	100	μg ml^{-1}	μM
α-Cotonefuran (**1**)	—	61	57	0	27	93
2	92	86	23	12	36	124
3	85	79	57	53	100	329
4	90	71	54	0	48	150
β-Pyrufuran	94	91	81	47	96	350

b. *Botrytis cinerea*

	Germination rate at (μg ml^{-1}) in percent				ED_{50}	
Compound	10	20	50	100	μg ml^{-1}	μM
α-Cotonefuran (**1**)	—	97	14	27	35	121
2	83	77	21	12	29	100
3	78	63	56	38	55	181
4	—	67	—	0	39	122
β-Pyrufuran	87	80	52	31	58	212

c. *Fusarium culmorum*

	Germination rate at (μg ml^{-1}) in percent				ED_{50}	
Compound	10	20	50	100	μg ml^{-1}	μM
α-Cotonefuran (**1**)	—	76	0	0	14	48
2	51	38	17	0	12	41
3	81	78	51	15	53	174
4	71	36	32	0	20	63
β-Pyrufuran	71	39	35	10	20	73

The results of these phytoalexin studies on *Mespilus germanica* confirm the generic status of this taxon, since three novel dibenzofurans **2**–**4** have been discovered in the infected sapwood of this plant. The presence also of α-cotonefuran (**1**) indicates that there is a weak affinity with *Cotoneaster*, and the structures of **2**–**4** suggest a possible affinity with *Pyrus*, which produces three related dibenzofurans. The data, however, rule out a close relationship with *Crateagus*, since in this genus, an unrelated dibenzofuran occurs as a constitutive antifungal agent [3]. In summary, the phytoalexin data generally support the conclusion of isoenzyme studies [1] that *Mespilus* is a natural unit distinct from other maloid genera in this group.

EXPERIMENTAL

The NMR data were obtained on a Bruker AMX 400 spectrometer.

Plant material examined was collected from the Harris Garden, the botanical garden of the University of Reading. Essentially the same procedures, for inoculation and incubation of the plant and measurement of ED_{50}, were used as described earlier [2, 3].

Isolation of phytoalexins. The thin shavings (220 g) of the necrotized tissue were extracted with MeOH for 1 week at room temp. The extract was filtrated and concd *in vacuo* and extracted × 3 with $CHCl_3$. The extract was concd and chromatographed on silica gel CC (2 × 33 cm, i.d.) with hexane–$CHCl_3$–Me_2CO [(1) 3:2:0, (2) 30:20:1 and (3) 12:8:1]. Biologically active frs were combined (syrup, 2.1 g) and sepd over silica gel prep. TLC with $CHCl_3$–EtOH (24:1). Three major antifungal bands (A, R_f 0.64, 153 mg; B, R_f 0.56, 480 mg; C, R_f 0.47, 707 mg) were further sepd: band A with hexane–EtOAc (3:2), band B with CH_2Cl_2–Me_2CO (49:1) and band C with hexane–EtOAc–MeOH (60:40:1). Compound **3** was obtained from the band A and further purified with toluene–EtOAc (4:1, R_f 0.53) over silica gel TLC. Band B gave the mixture of α-cotonefuran and **4** and they were sepd with $CHCl_3$–EtOH (97:3, R_f 0.50 and 0.42, respectively). The major antifungal band from band C was repeatedly chromatographed and **2** obtained.

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REFERENCES

1. Phipps, J. B., Weeden, N. F. and Dickson, E. E. (1991) *Syst. Botany* **16**, 546.
2. Kokubun, T., Harborne, J. B., Eagles, J. and Waterman, P. G. (1995) *Phytochemistry* **38**, 57.
3. Kokubun, T., Harborne, J. B., Eagles, J. and Waterman, P. G. (1995) *Phytochemistry* **39**, 1033.
4. Kemp, M. S., Burden, R. S. and Loeffler, R. S. T. (1983) *J. Chem. Soc. Perkin Trans. I* 2267.
5. Panichpol, K. and Waterman, P. G. (1978) *Phytochemistry* **17**, 1363.
6. Quader, A., Gray, A. I., Waterman, P. G., Lavaud, C., Massiot, G. and Sadler, I. H. (1991) *Tetrahedron* **47**, 3611.